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From Cellular Mechanotransduction to Biologically Inspired Engineering

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Abstract

This article is based on a lecture I presented as the recipient of the 2009 Pritzker Distinguished Lecturer Award at the Biomedical Engineering Society annual meeting in October 2009. Here, I review more than thirty years of research from my laboratory, beginning with studies designed to test the theory that cells use tensegrity (tensional integrity) architecture to stabilize their shape and sense mechanical signals, which I believed to be critical for control of cell function and tissue development. Although I was trained as a cell biologist, I found that the tools I had at my disposal were insufficient to experimentally test these theories, and thus I ventured into engineering to find critical solutions. This path has been extremely fruitful as it has led to confirmation of the critical role that physical forces play in developmental control, as well as how cells sense and respond to mechanical signals at the molecular level through a

process known as cellular mechanotransduction. Many of the predictions of the cellular tensegrity model relating to cell mechanical behaviors have been shown to be valid, and this vision of cell structure led to discovery of the central role that transmembrane adhesion receptors, such as integrins, and the cytoskeleton play in mechanosensing and mechanochemical conversion. In addition, these fundamental studies have led to significant unexpected technology fallout, including development of micromagnetic actuators for non-invasive control of cellular signaling, microfluidic systems as therapeutic extracorporeal devices for sepsis therapy, and new DNA-based nanobiotechnology approaches that permit construction of artificial tensegrities that mimic properties of living materials for applications in tissue engineering and regenerative medicine.

Keywords: Mechanotransduction, Tensegrity, Cell mechanics, Prestress, Cytoskeleton, Integrin, Biomimetic

INTRODUCTION

This article is based on the 2009 Pritzker Distinguished Award Lecture I presented to the Biomedical Engineering Society in the opening symposium of their annual meeting that was convened at Pittsburgh, PA in October 2009. My scientific education was limited to the fields of molecular biochemistry, cell biology and medicine, and I have never taken an academic course in engineering. Thus, virtually all of the contributions I have made in the field of bioengineering for which I am being honored today are based on what I have learned from my trainees, and from my many outstanding collaborators. To me, this is one of the greatest messages I can convey to young faculty: remember that you can learn as much (or more) from your students, as they can learn from you. Thus, I dedicate this lecture to all of the biomedical engineering students and fellows whom I have trained over the course of my career.

For the past thirty years, my laboratory has pursued the fundamental question of how living cells and tissues are constructed. This is an exciting and important question for engineers and material scientists because living cells are the ultimate “intelligent” materials. They are mechanically strong and resilient; exhibit integrated multifunctional chemical/mechanical/electrical/optical and information-processing capabilities; grow, move and self-heal; learn, adapt, and self-organize; and emerge through hierarchical self-assembly of nanoscale components. So for many years my group has strived to identify the design principles that Nature uses to build living cells, with the long-term goal of leveraging this knowledge to develop new biologically inspired materials and devices for Tissue Engineering and Regenerative Medicine.

THE LIVING CELL AS A MECHANICAL STRUCTURE

Most people believe that living cells are built from an elastic membrane that encloses a viscous or viscoelastic cytoplasm with a nucleus at its center, much like a balloon filled with molasses or jello. However, it is very difficult, if not impossible, to explain how cells do all the myriad tasks they do to build tissues and organs in the embryo, and to

maintain our bodies throughout adult life, if they were built this way. This is because development is an incredibly physical process in which cells pull and push and twist on each other until the interacting cells and tissues deform, bend, and fold into the characteristic three-dimensional (3D) forms that make up the typical body plan of every organism. Moreover, all adherent cells generate tensional forces within their internal cytoskeleton through an actomyosin-based contraction mechanism. Cells exert these forces on their adhesions to other cells and to extracellular matrix (ECM) scaffolds composed of various types of collagens, proteoglycans, and large glycoproteins (e.g., laminin, fibronectin) that hold cells together within specialized tissues. Cells lose their characteristic shapes (e.g., cuboidal, columnar, pyramidal) and round when they are dissociated from living tissues using ECM-degrading enzymes (e.g., collagenase, proteases), and they spread by applying traction forces to their new adhesions when cultured on tissue culture substrates that are coated with ECM molecules or serum-components.

These internal forces that govern cell adhesion and control cell shape are hidden in cultured cells and in cells within living tissues because they are isometric, that is, they are balanced by opposing forces due to the rigidity of the adhesion substrate (e.g., culture dish or ECM), and to the pulling forces of neighboring cells. This internal resting tension or "prestress" can be visualized by culturing cells on flexible substrates, such as silicon rubber, which fold up into compression wrinkles when the cells exert traction forces on their basal ECM adhesions. The existence of these micro-scale forces is important because it is well known that physical forces acting at the organ level can significantly influence the function of cells within living tissues. A few examples include the effects of compression on bone, tension on muscle, pressure on lung, and fluid shear on blood vessels.

A STRANGE IDEA: TENSEGRITY IN CELLS

So how are cells constructed so that they can generate, sense, and respond to physical forces? When I was a graduate student, I proposed an alternate hypothesis to the water balloon model of cell structure; instead, I suggested that cells might be built more like tents. In order to stabilize a tent, we need to prestress the flexible nylon fabric by placing it under tension. We do this by anchoring it to the ground at multiple sites using tent pegs, and then pushing the membrane up against these adhesions using tent poles. My vision at that time (in the late 1970s) was based on the then recent discovery that all adherent cells contain a contractile cytoskeleton, which is made up on a network of molecular cables, ropes, and struts that span from the nucleus to the surface membrane. Many biologists have studied the role of the cytoskeleton with the belief that it is critical for control of cell growth and movement; however, they tend to focus on its gel properties, as if it were truly organized like jello. In contrast, I suggested that living cells use a particular form of architecture that depends on tensional integrity to convey shape stability, and is known as "tensegrity".[16,24,25]

Most man-made constructions, such as Stonehenge, gain their stability from continuous compression, as in brick-upon-brick type constructions in which gravitational forces compress one building element down upon another. These are generally stable structures; however, if there is an impact from the side, the individual building components can fall like dominoes. By contrast, tensegrity structures are composed of a network of tensed elements linked to a subset of

elements that resist being compressed, and thereby bring the entire system into a state of isometric tension. The architect Buckminster Fuller first coined this term. His student, the sculptor Kenneth Snelson, was the first to reduce this concept to its simplest embodiment by physically constructing sculptures composed of stainless steel beams interconnected by tension cables that hold themselves stable against the force of gravity even though the beams never come in direct contact.

Even though geodesic domes can be constructed using all solid struts, Fuller realized that the reason his domes were such efficient structures in terms of their capacity to bear loads with a minimum of building materials was because they utilized tensegrity principles to ensure their stability. Interestingly, the first article published using anti-actin antibodies to visualize the cytoskeleton in cultured non-muscle cells described the presence of actin geodomes—miniscule geodesic domes with nanometer-sized struts, each composed of many parallel actomyosin filaments.[27] Thus, the suggestion that cells might use tensegrity architecture did not seem that outrageous. As part of my Ph.D. dissertation, I built tensegrity models of the cell composed of sticks and elastic strings.[16,24] I found that when the tensed structure was unanchored, it took on a round form much like a living cell because tensional forces pulled inward on all sides, with only internal struts providing resistance. However, when I pegged the structure to anchoring points distributed across a rigid substrate, the same cell model extended and took on a flattened and spread form, much like a cell when it adheres to culture substrate. And when I clipped these anchors, the model spontaneously retracted and leapt up off the substrate, again closely mimicking the behavior cells exhibit when trypsinized from a culture dish. This was enough for me conceptually: cells must be tensegrity structures, but it would take me, the members of my laboratory team, and multiple collaborators the next thirty years to provide experimental evidence to convince others that there was indeed substance to this theory.

ENGINEERING APPROACHES PROVIDE NEW INSIGHTS INTO CELL STRUCTURE

My group and many others have been able to demonstrate that many types of cells behave mechanically like tensegrity structures. For example, we confirmed experimentally that large bundles of actomyosin filaments, called stress fibers generate tension and experience tensile prestress in living cells.[26] We accomplished this using a laser nanosurgery technique that we developed in collaboration with Eric Mazur (Department of Physics, Harvard University). By focusing light generated by a femtosecond laser through a microscope objective, we could effectively generate the heat of the sun within a tiny volume for an incredibly short time (10^{-15} s), thereby vaporizing all molecules located within this location. When we focused the laser on a single stress fiber labeled with green fluorescent protein (GFP) in a living cultured cell, we produced a circular punch hole with a 300-nm diameter, which spontaneously deformed into an elliptical form (with long axis in parallel with the fiber), thereby directly visualizing tensile prestress within this structure.[26] In addition, when we severed a stress fiber in a spread cell attached to a rigid glass substrate, both cut ends retracted spontaneously, confirming that these are tensed structures; however, none of the other stress fibers in the same cells changed their shape or orientation. But when the same experiment was carried out with cells cultured on a flexible ECM-coated polyacrylamide substrate that had a compliance closer to that of living tissues, global structural

rearrangements were observed throughout the interconnected actin cytoskeleton, much like when a single tensile fiber is cut in a tensegrity sculpture.

We also have been able to show that microtubules can bear compression in living cells, and that they resist inward-directed forces generated by the surrounding contractile actin cytoskeleton.[3,55] For example, working with Kit Parker (School of Engineering and Applied Sciences, and Wyss Institute, Harvard University), we demonstrated that when cultured cardiomyocytes are transfected with GFP-tubulin, their microtubules buckle in unison when these beating heart cells contract, and they straighten when the cells relax.[3] Interestingly, if heart cells have too many microtubules, they mechanically interfere with contractility, and this can lead to heart failure in whole animals.[52] Most importantly, different types of cells exhibit the same microtubule buckling morphology with a wavelength of approximately 2–3 μm, and working with David Weitz (Department of Physics, Harvard University) and Fred Macintosh (Vrije Universiteit, The Netherlands), we showed that a constrained buckling theory which assumes that microtubules bear compression in living cells can quantitatively predict this curvature.[3] This is because it is energetically more efficient for microtubules to buckle into many small curves (i.e., rather than to undergo Euler buckling with a single large bend) when the microtubule is pushing out against a surrounding viscoelastic cytoskeleton. Interestingly, these studies revealed that microtubules can bear more than 100 times higher levels of compressive loading when present within the living cytoskeleton, than previously assumed based on studies with microtubules in isolation.

These findings confirm that cell shape stability depends a mechanical force balance in which microtubule struts balance inward-directed compressive forces generated by tensile actomyosin filaments, as predicted by the tensegrity model. However, cells also anchor to the ECM substrate through multiple small discrete tethering sites, known as “focal adhesions,” which function much like tent pegs. This is where cells cluster together their integrin receptors, which mediate cell surface binding to various ECM proteins. The cytoplasmic tails of integrins bind to actin-associated molecules, such as talin, vinculin, paxillin, and α-actinin, and thereby recruit them to form the structural backbone of the focal adhesion that forms a molecular bridge linking ECM and integrins to the internal contractile cytoskeleton. These discrete focal adhesions form at the distal ends of each stress fiber, which attempts to shorten when their internal actomyosin filaments slide and generate tension; this results in an increase in cytoskeletal prestress and flattening in a cell adherent to a rigid substrate (that can resist filament contraction), whereas cells round and form compression wrinkles as these adhesions are pulled closer to each other (like a purse-string) when the cell is cultured on a flexible substrate. Thus, the cell’s ECM adhesions also can work in a complementary way with microtubules to resist inward-directed cell tensional forces, and forces can be shuttled back and forth between ECM, microtubules, and the actin cytoskeleton in living cells.[21]

INTEGRINS AS MECHANORECEPTORS

One of the most important facets of the cellular tensegrity model is that it led me to predict that transmembrane cell surface receptors, which physically link external support scaffolds (e.g., ECM, other cells) to the load-bearing

cytoskeleton of the cell, may function as mechanoreceptors.[15,24,25] In other words, they would be among the first molecules on the cell surface to sense a physical force, and they transfer this mechanical signal across the cell surface via a specific molecular pathway. Because integrins are a ubiquitous class of transmembrane receptors that physically couple ECM to the actin cytoskeleton, we set out to explore whether they could mediate mechanosensing in living cells.

In order to test this hypothesis, we developed various magnetic cytometry techniques in which small (1–10 μm diameter) magnetic beads are coated with ligands for integrins (e.g., ECM proteins, RGD peptide, specific antibodies) or other transmembrane receptors, and then applied magnetic fields are used to either twist (shear) or pull (tense) on these specific receptors through the bound beads.[36,37,54] We found that when we applied force to transmembrane receptors, such as metabolic receptors or histocompatibility antigens, which do not support focal adhesion formation or mechanically couple to the deep cytoskeleton, and that these bead adhesions were very flexible as expected from the submembranous actin–spectrin–ankryn lattice to which they interconnect. However, when the same forces were applied to integrins that form focal adhesions on the same cells, the beads exhibited great resistance to pulling or twisting that increased in direct proportion as the level of applied stress was raised. Moreover, working with Dimitrije Stamenovic (Department of Biomedical Engineering, Boston University), we developed a mathematical tensegrity model starting from first mechanistic principles that predicted these results, and led to quantitative and qualitative predictions of many other mechanical behaviors of various types of living cells, and similar results were obtained by other investigators around the world.[21,50,51] Most other models of cell mechanics are *ad hoc* models, and while they can predict some of these behaviors, only the tensegrity model can predict this huge range of different cellular responses *a priori*. Thus, it appears to be the most universal model of cell structure at the present time.

STRUCTURAL HIERARCHIES FOR FORCE TRANSMISSION IN LIVING SYSTEMS

One of the most interesting properties of tensegrity structures first recognized by Buckminster Fuller is that hierarchical tensegrities may be built, which exhibit force transmission and fine structural coordination across multiple size scales.[12] This is important because one of the major differences between living systems and man-made structures is that while man usually builds using bulk materials (e.g., a hunk of metal or rubber), Nature builds hierarchically. For example, scientists can separate a nucleus from the cytoplasm of a living cell, and then transfer that nucleus to a second enucleated cell to create a fully functional organism, as in the cloning of "Dolly the Sheep." This means that the nucleus has its own structural and functional integrity that is maintained when it is isolated, as does the cytoplasm, and yet when you recombine them and these structures reconnect, normal structures and functions are restored.

I explored this idea many years ago by constructing a tensegrity cell model containing a nucleus at its center, which was connected to the surface of the model by additional elastic strings (i.e., to provide tensional integrity across size scales).[16,24] Now, when the model was unanchored, both the cell and nucleus exhibited round forms, but when the surface of the model was tethered to a rigid substrate, both the cell and nucleus spread and flattened in a coordinated manner due to the presence of these tensional connections. Moreover, when the adhesive anchors were clipped, both the

cell and nucleus rounded as well. Importantly, we demonstrated precisely this type of coupling between cell and nuclear spreading within living mammalian cells years later.[14]

In order to experimentally confirm that tensile connections between the nucleus and cell surface exist in living cells, we coated a glass micropipette tip with the ECM protein fibronectin and bound it to integrins on the surface of a cultured cell; then a micromanipulator was used to rapidly pull on these receptors. Amazingly, we not only found that this resulted in coordinated distortion of the surface membrane and nucleus, but also detected molecular realignment within nucleoli (using birefringence microscopy) within the center of the nucleus when we pulled on integrin receptors tens of microns away on the surface membrane.[34] Importantly, when we pulled on other transmembrane receptors in the same cells, only a local response at the membrane was observed. By carrying out similar studies in cells treated with pharmacological inhibitors of intermediate filaments, or in which the intermediate filament protein vimentin was knocked out genetically, we confirmed that intermediate filaments that have been previously shown to span from the nuclear surface to the surface membrane are largely responsible for this form of transcellular mechanical coupling.[9] However, the actin cytoskeleton also contributes to this coupling because pulling on integrins in mitotic cells that lack an intermediate filament cytoskeleton can actually cause reorientation of the mitotic spindle axis.[9] But these cells tear when pulled on and the cytoskeleton experiences more than about 15% mechanically strain. This finding emphasizes how studying the gel-like behavior of the actin cytoskeleton alone is not sufficient to explain the mechanical properties of living cells.

The observation that forces applied to surface integrins result in reorientation of nucleoli suggested that the nucleus itself must contain some type of load-bearing structural network. In fact, we were able to confirm the existence of mechanical connectivity within the entire living human genome by using an ultrafine micropipette (500-nm tip) to harpoon a single chromosome in a living mitotic cell, or a single nucleolus in an interphase cell.[33] Pulling a single chromosome out of a cell resulted in progressive removal of all of the remaining chromosomes, and when one nucleolus was extracted, it similarly resulted in removal of the entire interphase chromatin along with all of the remaining nucleoli. Importantly, cells remained viable during this nanosurgery procedure, and, in fact, we have used this extracted mitotic genome to study the physiology of chromosome decondensation and recondensation.[2] The key point here is that all of the chromosomes are linked by a continuous elastic network in living mammalian cells.

It has been long known that mitotic spindles can be isolated from living cells, and that they maintain their mechanical stability when isolated free of cells and studied *in vitro* even though they are made of molecules that we commonly think are dominated by thermal noise. Apparently, the stiffened microtubules stabilize the spindle by pushing out against this surrounding elastic chromatin network, and hence creating a tensegrity force balance on a smaller scale. Pickett-Heaps and his group in Australia has confirmed that the spindle microtubules bear compression: when he severed one microtubule with a laser, the others buckled, thus demonstrating the shift in compressive force to the remaining semiflexible microtubular struts.[44] But it doesn't stop here. We recently showed that individual actin stress fibers can be modeled as tensegrity structures because they are composed of actomyosin filaments that generate tension by

shortening, but the stress fiber does not narrow when tensed.[30] Hence, some of the other molecular components of the fiber must resist being compressed laterally to stabilize the entire tensionally prestressed fiber structure. Moreover, this tensegrity model again was able to predict a wider range of mechanical behaviors of stress fibers than any other existing stress fiber model.[30]

Amy Sung and Bob Skelton (Department of Biomedical Engineering, UCSD) independently showed that mechanical behavior of the red blood cell membrane can be predicted using a tensegrity model.[53] In their model, the geodesic array of spectrin molecules within the submembranous cortical cytoskeleton form the tensile network of the tensegrity structure, whereas the relative stiff actin protofilament and intervening region of the non-compressible lipid bilayer at the surface membrane act as compression elements. This model helps to explain why prestress is critical for shape stability of the erythrocyte membrane, and it emphasizes how tensegrity structures can be built with materials other than sticks and strings or struts and cables, and still provide this form of shape stability. Pursuing this concept even further, I suggested some years ago that individual molecules might stabilize their shape through use of tensegrity principles.[19] For example, proteins have multiple rigidified domains (e.g., α-helices, β-strands) interconnected by flexible regions, and multiple portions of the molecule pull in on themselves through attractive hydrogen bonding to assume a stable form. Moreover, we know that proteins are prestressed because enzymatic cleavage of the protein backbone often results in the molecule splaying open (i.e., loss of shape stability). Tensegrity structures are notorious for their ability to produce global rearrangements throughout the structure in response to local force application. Thus, this might explain why binding or pulling a small region of a large transmembrane surface receptor molecule (e.g., growth factor receptors, integrins, cadherins) can result in coordinated structural rearrangements throughout the molecule, thereby inducing shape transitions at its cytoplasmic face, which mediate transmembrane signaling.

Artists also have described the human body as a tensegrity structure. Each of us has hundreds of individual bones that are connected by a continuous tensile network, composed of muscles, tendons, ligaments, and fascia, which pulls us up against the force of gravity and stabilizes our forms. The stiffness of our bodies is not controlled by osmotic forces; rather it is the tensile prestress or tone in our muscles, which governs whether we stand strong or move in response to stress, much like in a tensegrity model. These observations bring home the point that Nature uses tensegrity principles to stabilize the structural hierarchies of molecules, cells, tissues, and organs that comprise our bodies.[19,21] The use of tensegrity also provides a mechanism to transfer mechanical signals from the macroscale to the nanoscale by transmission across tensional connections, and it facilitates mechanical integration between part and whole.[21,22] Thus, the structural organization of our bodies determines how physical forces will be sensed and received at all size scales.

SOLID-STATE BIOCHEMISTRY AND CELLULAR MECHANOTRANSDUCTION

The body's structural hierarchies enable it to channel mechanical forces applied at the organ level through load-bearing prestressed networks of bones, muscles, tendons, fascia, and ECM scaffolds so that they impact cell and molecular structure at the nanometer scale.[22] This is important because the cytoskeleton that gives cells their shape is more than a

mechanical scaffold; it also orients most of the cell's metabolic machinery. Many of the enzymes and substrates that mediate DNA synthesis, RNA processing, transcription, translation, glycolysis and signal transduction do not function in solution, or when floating free in the lipid bilayer. Instead, cells use a form of "solid-state" biochemistry in which these components function when immobilized on the insoluble scaffolds that comprise the cytoskeleton and interlinked nuclear matrix.[17]

Moreover, these chemical reactions are often "channeled" through these structures so that substrates are not freely diffusible and pass from one modifying enzyme or enzyme complex to neighboring functional assemblies. Because these reactions are not diffusion limited, cells can exhibit much higher efficiencies of biochemical reactions than we can mimic in solution in a test tube. Thus, use of solid-state biochemistry, in which the cytoskeleton is both a mechanical structure and a catalytic assembly simultaneously, is one of the most fundamental design principles used by living cells, and this insight into cellular engineering might throw some light into how life first originated on this planet.[20]

This coupling between structure and biochemistry within the cytoskeleton also provides a mechanism for cells to convert external mechanical signals into changes in intracellular biochemistry and gene expression—a process known as cellular mechanotransduction.[15,18,22] This is because physical distortion of biomolecules changes their thermodynamic and kinetic properties, and hence, alters their biochemical activities. For example, stress-sensitive ion channels alter their opening and closing rates (kinetics) when tensed or strained, either directly or through their interconnections with underlying cytoskeletal scaffolds. Other molecules, such as myosin motors and RNA polymerases, have been shown to alter their biochemical functions when physically distorted by piconewton scale forces applied using optical tweezers. Certain proteins, such as fibronectin, physically unfold when cells apply tension to their matrix adhesions and expose new binding site within the molecule the promote ECM fibrillogenesis. And cytoskeletal microtubules alter their polymerization dynamics when mechanically stressed such that they depolymerize when compressed and assemble when decompressed.

This coupling between mechanics and biochemistry at the nanoscale led me to suggest many years ago that stress application to transmembrane receptors that physically couple the ECM to the actin cytoskeleton—as integrins do in focal adhesions—might mediate mechanochemical conversion in living cells.[15,24] Importantly, the cytoskeletal backbone of the focal adhesion also orients many of the cell's signal transduction molecules, including protein tyrosine kinases (e.g., src, FAK), small and large G proteins, ion channels, mediators of inositol lipid signaling, and a subset of growth factor receptors (e.g., FGF receptors), among others.[18,24] Thus, given that we had previously shown that mechanical forces are preferentially transmitted across integrins that directly connect with these focal adhesions, it seemed likely to be a potential site where mechanochemical conversion might occur.

My group explored this possibility by adapting the magnetic twisting and pulling cytometry techniques we had developed previously to apply controlled mechanical stresses to cells while quantitating changes in cellular biochemistry and gene expression. Using this approach, we confirmed directly that cell surface β1-integrin receptors

mediate mechanically induced changes in cellular mechanotransduction. For example, we showed that applying shear or tension to integrin-bound magnetic beads stimulates the entire cAMP-signaling cascade, as measured by activation of large heterotrimeric G proteins within the focal adhesions, increased production of cAMP by adenylyl cyclase, enhanced nuclear translocation of the catalytic subunit of protein kinase A, activation of the transcription factor CREB, and induction of reporter gene transcription driven by multiple cAMP response elements.[1,39] Again, application of the same stresses to other transmembrane receptors, such as metabolic receptors that do not form focal adhesions, failed to produce any of these responses. We also have used this approach to demonstrate that integrins mediate stress-induced changes in mRNA and ribosome distritribution[5] as well as calcium signaling[37] in living cells. In our most recent unpublished studies, we have been able to detect mechanical stress-dependent increases in calcium influx into cells at the focal adhesion site where magnetic beads bind to integrins on the cell surface within 5 ms after tension is applied to these beads using applied magnetic forces (Matthews *et al.*, in review). To our knowledge, this is the most rapid integrin-specific mechanochemical conversion event that has been demonstrated in living cells.

Thus, integrins are indeed mechanoreceptors that transmit mechanical forces across the cell surface over a specific molecular pathway, and that facilitate mechanochemical signal conversion inside the cell. These studies combined results from other leading researchers in this area (e.g., Burridge, Geiger, Horwitz, Schwartz, Sheetz, Yamada, among others) have led to the recognition that the focal adhesion functions as a nanoscale mechanochemical machine that mediates cellular mechanotransduction. Forces applied at the macroscale that are channeled over stiffened ECM elements to surface integrin receptors result in distortion of focal adhesion molecules. Changes in molecular shape can alter the biochemical activities of these molecules in many ways, including altering binding/unbinding rates (e.g., zyxin),[28] unfolding proteins and exposing phosphorylation sites (e.g., p130Cas),[46] altering cytoskeletal filament assembly,[45] modulating ion flux,[37] or through some other mechanism that remains to be identified. Conversely, application of cell traction forces on integrins and surrounding ECM can feed back to alter biochemistry outside the cell, for example, by unfolding fibronectin proteins and promoting ECM fibril formation.[62] In this manner, changes in cytoskeletal tension and cellular prestress can alter both biochemistry and structure in a highly coordinated manner, which is critical for cell and tissue development.

But there is more to cellular mechanotransduction than local mechanical signaling across at the focal adhesion. One of the novel predictions of the nucleated cellular tensegrity model is that forces applied to integrins at one point on the cell surface can be rapidly channeled across interconnected cytoskeletal elements to multiple locations distributed throughout the entire cell and nucleus.[16,24] As described above, we demonstrated this type of "action-at-a-distance" many years ago by pulling on integrins with ECM-coated micro-pipettes and inducing changes in nuclear and nucleolar structure inside the cell.[34] We also demonstrated that intermediate filaments and actin filaments are required for this response.[9,34] However, recent studies by others has identified the LINC protein complex on the surface of the nucleus composed of Nesprin, Sun, and Emerin proteins, which appears to physically couple cytoskeletal filaments in the cytoplasm to the nuclear surface and lamins within internal nuclear scaffolds that, in turn, link to genes and their regulatory proteins.[56]

Most importantly, long-distance force transfer across the prestressed cytoskeletal framework enables transcellular mechanical signaling to occur much more rapidly than chemical signaling. For instance, Ning Wang (Department of Biomedical Engineering, University of Illinois Urbana-Champaign) has shown that force application to magnetic beads bound to surface integrins activates the protein tyrosine kinase, c-Src, at distant sites within the cytoplasm within 0.3 s after force application, and this response can be suppressed either by disrupting the actin cytoskeleton or merely by altering the level of prestress in the cytoskeleton.[41] Thus, this fundamental principle of tensegrity—the dependence of mechanical stability on tensile pre-stress—is also a key feature of cellular mechanochemical conversion.[51]

MECHANICAL CONTROL OF CELL FUNCTION AND TISSUE DEVELOPMENT

My belief that cells are tensegrity structures led me as a graduate student to think about embryological development and tissue formation from a mechanical perspective. Early biologists at the beginning of the last century had described tissue development in largely mechanical terms; however, interest in this area waned with the advent of biochemistry, and it virtually died once the power of molecular biology and genetic engineering was recognized. Yet, there was no way to ignore the reality that embryological development is a highly mechanical process, and that it is impossible to explain movement or changes in form at the micro and macro scale without employing physical forces.

If cells are prestressed tensegrity structures, then tissues also must be prestressed. In fact, we know this to be true; this is why surgeons must suture a wound to ensure tissue closure. If a developing tissue, such as a budding epithelium, exhibits a stable form, then the tensional forces exerted by the epithelial cells must be balanced by opposing mesenchymal cells in the stroma below, and by the planar ECM or basement membrane that separates these tissues. If this ECM is tensed due to the traction forces surrounding adherent cells exert on it, then local breakdown or thinning of its structural backbone will cause it to stretch out, much like a “run” in a woman’s stocking. Interestingly, local thinning of the basement membrane due to localized elaboration of matrix-degrading enzymes occurs at virtually all sites where new epithelial bud and capillary branches will form in developing tisssues before cell growth ensues. A seminal article in 1978 by the late Judah Folkman had suggested that cell shape is tightly coupled to growth, with spread cells growing more rapidly than round given the same soluble cues.[11] Thus, this led me to propose a micromechanical mechanism for morphogenetic control in which changes in ECM remodeling alters ECM mechanics (e.g., flexibility), which promotes cell stretching and thereby alters local cell growth.[13,24] Although I had no idea of a molecular mechanism at that time, I suggested that increased tension on the ECM receptors of the cells adherent to these thinned regions of the tensed basement membrane would induce cytoskeletal distortion and thereby alter cellular biochemistry, somehow leading to the localized changes in cell proliferation and motility, which drive changes in tissue form. We, therefore, set out to explore whether cell shape distortion influences cell growth and function. In an earlier study, we showed that cell spreading and growth increase in parallel when anchorage-dependent cells are cultured on increasing molecular coating densities of ECM molecules, such as fibronectin, laminin, or different collagen types.[14,23] However, increasing ECM molecular densities also promotes integrin clustering, which we found can induce chemical signaling independently of cell shape distortion.[48] Thus, in order to unequivocally address the question of cell shape in growth control, we set out

to develop an experimental system in which cell shape can be varied independently of other critical control parameters.

In order to accomplish this, we desired to develop an experimental system in which individual cells are cultured on single cell sized adhesive islands coated with a high ECM coating density (i.e., which promotes optimal integrin clustering) surrounded by non-adhesive barrier regions. The concept was that cells adhere and spread by applying traction forces to their ECM contacts and thus, cells would spread to take the shape of the adhesive island until they reached the non-adhesive edge that could not resist cell traction forces. In this manner, plating a cell on large circular ECM island should result in a pancake-shaped cell, whereas on a smaller island, the cell would be shaped more like a cupcake, and on a tiny island, it would take on the form of a golf ball-on-a-tee. If we cultured the cells in chemically defined medium containing a saturating amount of growth factors and they all adhered to the same high density of immobilized ECM protein, then the only experimental variable would be the degree to which cells physically distort and change shape.

We reduced this concept to practice by collaborating with George Whitesides (Department of Chemical Biology and Wyss Institute, Harvard University) who had recently developed the soft lithography-based microcontact printing technique as an inexpensive alternative to create microchips for the computer industry.[57] This method uses photolithography to create a master surface containing micrometer-sized indentations etched to take on the form, size and distribution of the final desired adhesive islands. Then an elastomer, polydimethylsiloxane (PDMS), is polymerized on its surface. When peeled off, the PDMS functions as a flexible rubber stamp that retains the surface topography down below the 100-nm scale. We inked the elevated regions of the stamp corresponding to the desired adhesive islands with alkanethiols, and transferred them in this precise pattern to gold-coated silicon chips or glass slides by pressing the stamp to the surface. The intervening regions between the islands were then made non-adhesive by adding soluble alkanethiols linked to polyethylene glycol molecules, which similarly bound to the gold-coated substrates and filled in all the spaces of the surface creating a completely planar, near-crystalline, self-assembled monolayer that supported ECM protein adsorption only in the regions corresponding to the desired cell adhesive islands.

Using this method, we showed that when capillary endothelial cells are cultured on large 50-μm diameter circular islands they indeed take on the shape of flat round cells, and when plated on small 10-μm circles, they appear almost spherical in form. In order to control for the total amount of ECM the cells contact, we created substrates in which the 10-μm circular islands were broken up in many tiny 3–5-μm diameter circular islands distributed so that cells spread from dot to dot over multiple islands. Thus, cells on these latter substrates spread like the cells on large ECM islands, yet they only adhered to small total area of ECM similar to that contacted by cells anchored to single 10-μm circular islands. In separate studies, we cultured liver epithelial cells on rectangular islands, and these cells took on the precise shape of the islands. These studies revealed that as island size and cell spreading were increased, DNA synthesis (as measured by entry into S phase) was promoted in a coordinated manner, regardless of the ECM contact area.[4,49] Moreover, when cell spreading was inhibited on smaller islands and growth as shut down, cells were induced to undergo apoptosis even though they were still anchored to the ECM substrate.[4] In addition, when capillary endothelial cells were

cultured on linear substrates that supported only a moderate degree of cell spreading as well as cell–cell interactions, differentiation was induced as indicated by formation of hollow capillary tubes within a period of hours.[7] Similar switching from growth to differentiation was seen in single liver cells when their spreading was partially inhibited using a similar approach.[49]

In later studies, we showed that when cells cultured on square ECM islands are stimulated with motility factors, they preferentially extend new motile process (e.g., lamellipodia, filopodia) at their corners, whereas cells cultured on similar ECM-coated circular islands exhibit no directional bias.[43] This is important because biologists interested in control of cell motility focus on identification of signaling molecules that are critical for movement, such as the small GTPase Rac. But while molecules such as Rac may be necessary for lamellipodia formation, they are activated to similar levels in different shaped cells, yet the physical microenvironment and direction of cell distortion apparently govern the way in which these signaling molecules manifest their motility-promoting activities.

More recently, my group has shown that this spatial control of motility signaling is controlled mechanically. Cells cultured on square ECM islands spread more along the diagonals (to reach the corners) than they do to the sides. This geometry results in stress concentrations at the cells' corners, which promotes focal adhesion formation in these regions and further increases cell prestress through activation of the small GTPase Rho.[43] This increase in tension promotes actin cytoskeletal remodeling, resulting in formation of contractile stress fibers oriented primarily along the diagonal axes of the cell, which in turn further enhances tension generate at their insertion sites on focal adhesions in the corner regions. Importantly, using a Rac-FRET construct, we experimentally confirmed that Rac is activated locally at sites of new focal adhesion formation within 1 min after cell surface binding to ECM substrates.[60] Thus, because cells carry out solid-state biochemistry on their cytoskeleton, physical forces that distort cell shape through their ECM adhesions that link to the cytoskeleton can impact the spatial distribution of signaling events inside the cell, and this appears to be how physical cues influence the direction in which cells will move.

Later study by Chris Chen and by Dennis Discher (both of Department of Bioengineering, University of Pennsylvania) extended these findings to show that cell shape distortion, cytoskeletal prestress, and ECM mechanics regulate mesenchymal stem cell (MSC) lineage switching. When human MSCs are cultured on microfabricated ECM-coated islands in the presence of a mixture of soluble inducing factors, virtually all of the spread cells on large islands differentiate into bone cells, whereas all of the round cells on small islands turn into fat cells.[38] Moreover, this process is mediated by RhoA-dependent cytoskeletal tension generation. In addition, when the same human MSCs are cultured on ECM-coated polyacrylamide substrates with different mechanical compliances that match those of living tissues, cells on rigid ECMs that mimic bone osteoid differentiate into bone cells, cells on less rigid substrates that mimic muscle form skeletal muscle cells, and MSCs on ECMs with the highly flexible properties of brain tissue become nerve cells.[10] Thus, cell-generated tensional forces, ECM mechanics, and cell shape distortion appear to play a fundamental role in developmental control.

In later studies on cell shape-dependent growth control, we discovered that the small GTPase Rho is also central to the mechanism by which mechanical distortion of cells and the cytoskeleton regulate the G1/S control point during cell cycle progression.[32] Rho's effects were mediated in large part by its downstream target, Rho associated kinase (ROCK), that increases cytoskeletal tension (prestress). With this molecular handle, we then set out to further explore the concept I had proposed years earlier[13,24] that changes in the balance of mechanical forces between the cytoskeleton and ECM could contribute to morphogenetic control *in vivo*. In order to do this, we carried out experiments in whole embryonic mouse lung rudiments, which contain epithelium and vascular endothelium that continue to undergo progressive budding and branching morphogenesis, respectively, when cultured *in vitro*.[40] When these developing lung rudiment were treated with the Rho activator, CNF-1, epithelial budding and capillary branching were both stimulated in parallel; however, when Rho levels were further activated with increased doses of CNF-1 and higher levels of myosin-based tension were induced, large scale contraction of the whole lung and growth inhibition were observed. In addition, when tension was dissipated using a ROCK inhibitor, budding morphogenesis and angiogenesis were both inhibited. Importantly, cytoskeletal tension suppression resulted in loss of pattern formation because regional thinning of the basement membrane and associated spatial differentials of cell growth were lost, and not because there was a generalized suppression of cell proliferation. Thus, these studies further support the concept that cytoskeletal prestress contributes significantly to morphogenetic control, as initially predicted by the tensegrity-based micromechanical model of developmental control.[13,24] More recently, we also confirmed that ECM mechanics is critical for angiogenic control *in vivo*, and identified two antagonistic transcription factors—TFII-I and GATA2—that sense changes in ECM compliance and regulate transcription of the gene that encodes the VEGFR2 receptor for the VEGF angiogenic factor.[31]

These findings also have great clinical relevance. For example, working with Dennis Orgill (Department of Plastic Surgery, Brigham and Women's Hospital and Harvard Medical School), we have analyzed therapies used to address the problem of large nonhealing skin wounds in humans.[47] One of the first growth factor therapeutics (PDGF) was developed and clinically approved for this application. It likely cost hundreds of millions of dollars to bring to the clinic, and although it works moderately well in patients, it is extremely expensive. Interestingly, one of the first tissue engineering scaffold therapeutics also was developed and approved for this medical application, and it too was costly to develop and is very expensive, yet it produces sub-optimal results.

Incredibly, the preferred therapy now used to treat this problem in the clinic is a simple mechanical therapy, called a VAC Sponge. It involves placement of a microporous biocompatible sponge in the wound, which is covered with an air-tight silicone seal and then suction is repetitively applied to the wound. This was initially developed with the goal of removing edema fluid; however, recent study suggests that the suction results in cyclical mechanical strain of the cells located within the interstices of the sponge, which is at the appropriate level to enhance the cells' ability to respond to growth factors and ECM proteins that already exist within these wounds.[47] Thus, this is a simple example of how a mechanical perspective can lead to a simple and a more cost-effective solution to a long-standing medical problem of great clinical importance.

Taken together, these studies clearly demonstrate that integrins, ECM, and the cytoskeleton control tissue development by mediating cellular mechanotransduction. Although genes, chemicals, and hormones regulate developmental responses, mechanical forces guide these processes and help to establish pattern formation in 3D. This novel insight into developmental control would not have been possible without the application of engineering approaches to confront fundamental questions in biology, and specifically melding these techniques with methods from molecular and cellular biology. I hope that it has been clear from my presentation so far that the problem is too large to be solved by any single existing discipline on its own.

BIOINSPIRED TECHNOLOGY FALLOUT

Although my group has long been interested in fundamental mechanisms of biological control, we also always seek to discover new ways we might apply the new insights we make or the tools we develop. This is an important lesson for all graduate students and fellows in Bioengineering, and thus, before ending I would like to present a few examples of how deeper appreciation of the way Nature builds, controls, and manufactures has led us to create new technologies that could potentially transform medicine in the future.

Our study on mechanotransduction revealed that application of mechanical stresses to cell surface molecules can influence biochemistry independently of classic receptor signaling mechanisms. But even chemical signaling mechanisms potentially involve mechanical forces. For example, many growth factors, such as EGF and PDGF, must cluster their receptors on the cell surface to elicit chemical signals inside the cell. This is normally accomplished chemically through multivalency; however, this is essentially a mechanical process as the receptors are physically dragged together on the surface membrane (in this case, driven by attractive forces due to binding to a multivalent ligand). We, therefore, explored whether physical forces alone are sufficient to activate this type of transmembrane signaling mechanism.

Because we had great experience with applying mechanical stresses to cell surface receptors through bound magnetic microbeads, we adapted this approach by coating much smaller magnetic nanobeads that were on the same size scale as individual receptor molecules (30 nm diameter) with a single ligand per bead for IgE receptors on the surface of cultured mast cells.[35] When these receptors normally bind to multivalent antigens, clustering of the IgE receptors induces calcium influx that stimulates histamine release. When we bound single magnetic nanobeads to one receptor each, they remained evenly distributed across the surface of these cells and did not stimulate signal transduction. However, when we applied an external magnetic field, the bound superparamagnetic beads magnetized and acting like small bar magnets pulled their neighbors into tight clusters. This physical clustering of the ligated receptors was sufficient to induce calcium influx inside the cell, and this process could be repeated in a cyclical manner by removing and applying the magnetic field from a distance.[35] This study revealed yet another way in which physical forces can influence cell behavior. However, it also represents a potentially exciting medical technology of the future in which cell contraction, nerve impulses, or gene expression could be controlled non-invasively inside our organs by applying a

magnetic “wand” over the surface of our bodies.

Another example of a medical technology that fell out from our basic study involves the application of microfluidics in biology. Around the same time, George Whitesides was developing soft lithography to microcontact print patterns on substrates, his group also pioneered the use of PDMS to engineer microfluidic systems that essentially mimic the microvasculature of living systems. Microfluidic systems are interesting because they only exhibit laminar flow due to their small size (fluid channels are usually <500 μm in largest dimension), and thus two different fluids introduced into a single flow channel through separate inlets do not mix and simply flow in parallel until they exit through two separate outlets. We took advantage of this novel feature of these microfluidic systems and combined it with our expertise in magnetic particles to develop a nanomagnetic-microfluidic cell separation device for sepsis therapy that effectively functions like an artificial spleen.[59,61]

Patients with sepsis have pathogens in their blood that spread throughout the body and often result in multiple organ systems failure and death. Although most pathogens remain within tissues, some patients respond fully to antibiotic therapy alone, which only decreases the pathogen load. If we could rapidly remove circulating pathogens while simultaneously treating with antibiotics, then we might be able to save increasing numbers of patients’ lives. In addition, if we could quickly remove these microbes from the blood, then we might be able to diagnose the specific type of pathogen immediately, and not have to wait many days to obtain results of blood cultures before tailoring their therapy appropriately. This is a particular problem in patients with fungal infection because anti-fungal therapy will not be initiated until a definite diagnosis has been made because the drugs have significant toxicity. Thus, we set out to develop an extracorporeal microdevice that could rapidly cleanse blood of pathogens without compromising or removing normal blood components that are critical to fight infections (e.g., immune cells, antibodies, complement, etc.). In order to accomplish this, we coat magnetic beads (130-nm or 1-μm diameter) with ligands for pathogens (e.g., antibodies to *E. coli* bacteria or *C. albicans* fungi), mix them with saline or whole human blood containing living pathogens, and then flowed the mixture through one inlet of a single channel microfluidic device while passing isotonic saline through the second inlet into the parallel flow stream. Without applied magnetic fields, the contaminated fluid and saline do not mix, and each pass out through their corresponding outlet. However, when a magnetic field is applied perpendicular to the direction of flow, the magnetic beads and bound pathogens are pulled across the flow border between the laminar streams, and they are removed through the isotonic saline outlet while the cleansed blood exits through its outlet and potentially can return back to the patient. We demonstrated greater than 85% filtration efficiency in our initial studies using *E. coli* in saline,[59] and more recently, and we have been able to cleanse whole human blood of *C. albicans* with greater than 90% efficiency at much higher flow rates.[59,61] These microdevices can be aligned in tandem and multiplexed to produce even greater efficiencies, and to cleanse larger volumes of blood in a clinical setting. Thus, we are pursuing this approach in animal studies for sepsis therapy; however, this represents a platform technology in that it also could be used to isolate many other types of circulating cells (e.g., cancer cells, stem cells, immune cells, fetal cells in the maternal circulation), molecules (e.g., antibodies, inflammatory cytokines), or any other component in blood or other biological fluid that would normally be restricted by a filtration membrane.

It was about the time we initiated these studies on the artificial spleen that we began to conceive of the concept of a research institute dedicated to development of technologies inspired by how Nature builds. This vision recently became a reality with the establishment of the Wyss Institute for Biologically Inspired Engineering at Harvard University that I now head. At the Wyss Institute, we have a major platform effort in Biomimetic Microsystems, which applies microengineering approaches to create "organ-on-a-chip" technologies that we hope will someday replace animals for applications in drug testing, toxicity, and device screening. One example of ongoing study in this area is the recent development of a "breathing human lung-on-a-chip" that recapitulates the functional alveolar–capillary interface of the lung, and exposes it to both fluid flows and cyclical mechanical strain similar to that observed *in vivo* (Huh *et al.*, in review). This device contains two parallel microfluidic channels separated by a porous membrane that is coated with ECM. It has human airway epithelium adherent on one surface, and human vascular endothelium adherent to the other; rhythmic suction is applied to the cells on the flexible membrane to mimic the distortion normally produced by the breathing motions of the lung. Interestingly, when living bacteria are placed in the alveolar channel of this device and blood containing fluorescently labeled neutrophils is passed through the vascular channel, these neutrophils can be observed to adhere to the inflamed endothelium and to undergo diapedesis as they migrate to the alveolar space where they engulf the pathogen. Hence, multiple stages of the inflammatory response that normally occurs *in vivo* can be studied at high resolution in this *in vitro* model. We also discovered that toxic effects of nanoparticles delivered to the alveolar channel can only be detected when the tissues experience physiological breathing motions. Thus, although these results are only preliminary, they strongly support the potential clinical value of engineering biomimetic systems that recreate functional tissue–tissue interfaces and experience physiological mechanical stresses.

Finally, one of the most fundamental biological design principles that we have uncovered is clearly the use of tensegrity architecture, which we observe at virtually all size scales in the hierarchy of life.[19,21,22] Over 10 years ago, I formed a company called Molecular Geodesics Inc. (later known as Tensegra Inc.) to leverage this knowledge to design and fabricate biomimetic materials that utilize tensegrity principles using computer-assisted design and manufacturing (CAD/CAM) techniques in combination with solid-free form fabrication strategies.[42] Unfortunately, methods did not exist to manufacture structures with the precise architecture we desired on the nanometer scale the way Nature builds. However, recent advances in nanotechnology now make it possible to build on this miniscule size scale and, in fact, self-assembled tensegrity arrays have been created using programmable DNAs that form defined structures based on complementary binding interactions. For example, William Shih (Dana Farber Cancer Institute, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School and Wyss Institute) has applied the DNA origami approach to build 3D tensegrities.[6,8] In DNA origami, a single circular strand of DNA is induced to fold up on itself and to create specific 3D forms (e.g., octahedral, icosahedra) much like twisting and folding a balloon animal, by allowing many small complementary DNA oligonucleotides to bind to sites located at different positions along the strand. Shih and his colleague Shawn Douglas have developed a CAD program (caDNAno) that allows one to define the oligonucleotide sequences to build virtually any 3D structure one desires on the nanometer scale.[58] Using this approach, we have worked together at the Wyss Institute to create a prestressed 3-strut tensegrity structure approximately 100 nm in length composed of a singular circular strand of DNA that folds up into nanotubular forms to create the struts, which

are interlinked by remaining linear regions of the same DNA strand.[29] Moreover, we can alter the prestress, and hence the mechanical stability in these structures, by designing different length nanotubular struts. Because DNA has the potential to be both biocompatible and biodegradable, we hope to extend this approach to create prestressed tensegrities that someday might be used as mechanical actuators to control ECM mechanics or regulate stem cell behavior for applications in tissue engineering and regenerative medicine. For any of you who would like to learn more about this research or the Wyss Institute, please explore our website (wyss.harvard.edu).

CONCLUSION

In this lecture, I tried to convey my path from science to engineering, and from fundamental mechanistic research to technology development and translation. I hope that I fully communicated the challenges I have faced as a biologist who believed that mechanics and structure are as important regulators of cell and tissue function as chemicals and genes. I had to seek out collaborators who shared in my vision and had the patience to teach me engineering principles, concepts, approaches, and vocabulary. But to me the key has always been to remain at the boundary between these disciplines because this is where the most exciting and dynamic advances always take place. In order to do interdisciplinary research and pursue unconventional ideas successfully, it is necessary to demonstrate to one's critics that you can use the same approaches they do, and obtain the same results. It is only when you have their respect as a peer, that you can go further and show that by looking at the problem from a slightly different perspective or using a new technology, you can explain things that they cannot, using existing approaches. It is only then that your critics become your competitors. Thank you once again for this wonderful honor.

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